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Solubilization and functional reconstitution of the protein-translocation enzymes of *Escherichia coli*

(secretion/liposomes/SecY/pro-OmpA/proton-motive force)

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ABSTRACT **The SecY protein and other membrane proteins of *Escherichia coli* were solubilized by mixed micelles of *n*-octyl β-D-glucopyranoside, phospholipids, and glycerol. Proteoliposomes formed from this extract by detergent dialysis supported energy-dependent translocation and processing of pro-OmpA. Translocation required ATP, SecY, and SecA and was stimulated by a proton-motive force. These results provide an important assay for the isolation and identification of membrane components involved in protein translocation.**

Translocation across inverted *Escherichia coli* inner membrane vesicles has been reconstituted with entirely purified soluble and peripheral membrane components (1, 2). *In vitro* translocation reactions (3, 4) provided a starting point for fractionation. Precursor proteins such as M13 procoat, pre-maltose-binding protein, pro-OmpA, and pre-PhoE have been purified (1, 5–7). Three cytosolic "chaperones," SecB, trigger factor, and GroEL, have been isolated and shown to stabilize precursor proteins for translocation (1, 8–13). SecA is the only peripheral membrane protein (14, 15) that is directly involved in translocation (2). Purified SecA hydrolyzes ATP when activated by translocation-competent precursor proteins (13, 16, 17), acidic phospholipids (18), and membrane-embedded SecY (16); this activity is referred to as "translocation ATPase" (16).

While there has been substantial progress in identifying and isolating cytosolic proteins involved in translocation, it has been difficult to reconstitute translocation-competent proteoliposomes from micellar detergent extracts of the membranes. Genetic studies have identified at least three integral membrane proteins encoded by *sec* genes: SecD (19), SecE (20, 21), and SecY (PrlA) (22, 23). No catalytic functions have yet been assigned to these Sec proteins. Reconstitution of an *in vitro* translocation reaction from all purified components may prove essential to resolve the functions of individual Sec proteins.

SecY can be solubilized by strong ionic detergents such as sodium dodecyl sulfate (SDS) (24) or deoxycholate (L. Brundage and W.W., unpublished data). Proteoliposomes reconstituted from deoxycholate extracts support the translocation ATPase but are unable to translocate pro-OmpA (L. Brundage and W.W., unpublished data). Nonionic detergents such as *n*-octyl β-D-glucopyranoside (OG) are optimal for chromatography and often preserve the biological activity of membrane proteins during extraction (25). However, SecY aggregates when solubilized in the presence of OG, though protein-translocation activity can be recovered from the particulate fraction (26).

The inclusion of glycerol (27, 28) and phospholipids (29) with nonionic detergents has a dramatic effect on the solubilization of transport proteins. We now report the solubilization of the integral membrane proteins involved in the translocation of pro-OmpA across the plasma membrane of *E. coli* and their functional reconstitution into proteoliposomes. Translocation into these proteoliposomes requires ATP hydrolysis and the SecA and SecY proteins and is enhanced by a proton-motive force (pmf).

MATERIALS AND METHODS

Materials and Bacterial Strains. Inverted inner membrane vesicles were prepared from *E. coli* strains D10 (1), KM9 (*unc*$^-$::Tn*10*) (2), and CJ107 (*secY24ts*) (30) by the procedure of Chang *et al.* (31). *E. coli* CJ107 was grown as described (32) to deplete SecY function. The SecA protein was purified as described (2). Pro-OmpA was prepared as described (1, 17) and dissolved in 6 M urea/1 mM dithiothreitol/20 mM Tris Cl, pH 8.0. Tran^{35}S-label, a mixture of 85% [^{35}S]methionine and 15% [^{35}S]cysteine (37 GBq/mol), was from ICN. [^{35}S]Pro-OmpA was synthesized *in vitro* and purified as described (11). Antiserum to the cytoplasmic N-terminal domain of SecY was described (26, 33). IgG was isolated from the antiserum (34).

Preparation of Liposomes and Bacteriorhodopsin Liposomes. Egg yolk L-α-phosphatidylcholine (Sigma) and acetone/ether-washed *E. coli* phospholipid (Avanti Polar Lipids), stored as chloroform/methanol (9:1, vol/vol) solutions, were mixed at a ratio of 1:9 (wt/wt) and dried under nitrogen and by lyophilization. Dried phospholipids were suspended at 20 mg/ml in buffer A (50 mM Hepes/KOH, pH 7.0/50 mM KCl/1 mM dithiothreitol), dispersed by bath sonication, and stored under nitrogen at −80°C. To prepare liposomes for reconstitution, frozen phospholipid stocks were thawed at 25°C and pulse-sonicated (0.5 sec) for 5 min on ice with a microtip (Heat Systems/Ultrasonics). Bacteriorhodopsin (bR) liposomes were formed by sonication (35, 36). Lyophilized purple membranes (38.4 nmol of bR) from *Halobacterium halobium* (Sigma) were added to preformed liposomes (10 mg of phospholipid) suspended in 500 μl of buffer A and the mixture was pulse-sonicated as described above.

Solubilization and (Co-)Reconstitution. The method used to solubilize inner membrane vesicles of *E. coli* with OG (29) was modified according to Ambudkar and Maloney (27). Membrane vesicles (0.4 mg of protein) were incubated in buffer A for 20 min at 0°C with 1.25% (wt/vol) OG (Calbiochem), 1.85 mg of preformed liposomes, and 20% (vol/vol) glycerol in a final volume of 500 μl. For the co-reconstitution with bR, liposomes were replaced by bR liposomes. After centrifugation (145,000 × g_{max}, 30 min, 4°C, Beckman Airfuge), the supernatant was dialyzed for 18 hr at 4°C against 1000 volumes of buffer A with one change after 4 hr.

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Abbreviations: bR, bacteriorhodopsin; OG, *n*-octyl β-D-glucopyranoside; pmf, proton-motive force.

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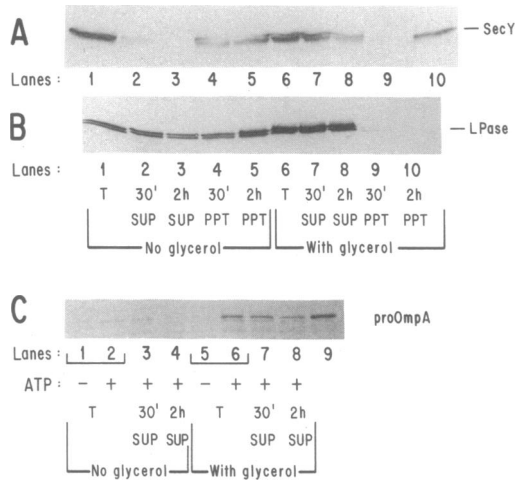

FIG. 1. Effect of glycerol on the solubilization of SecY and leader peptidase (LPase) and the recovery of translocation activity by proteoliposomes. (*A* and *B*) Membrane vesicles of *E. coli* KM9 were solubilized with OG and phospholipid in the absence or presence of glycerol. Extracts were fractionated by centrifugation (145,000 × g_{max}, 4°C, Beckman Airfuge) for 30 min or 2 hr, as indicated. Unfractionated extracts (T), supernatants (SUP), and pellets (PPT) were analyzed by SDS/PAGE, electrotransferred to Zeta-Probe membranes (Bio-Rad), and immunostained for SecY (*A*) or LPase (*B*). (*C*) Unfractionated extracts and supernatant fractions obtained after OG extraction in the absence (lanes 1–4) or presence (lanes 5–8) of glycerol were reconstituted by detergent dialysis and assayed for pro-OmpA translocation in the absence or presence of ATP (4 mM), as indicated. Lane 9 contained 5% of the input [^{35}S]pro-OmpA.

Proteoliposomes were fused by a single freeze–thaw–sonication cycle (37) and stored on ice.

Assay of Translocation ATPase Activity. Translocation ATPase was measured according to Lill *et al.* (18) in the presence of 5 mM $Mg(OAc)_2$. Control experiments performed with liposomes indicated that the contribution of SecA/lipid ATPase (18) activity to the signal was negligible (i.e., <2%) under these assay conditions.

Translocation Assay. Translocation of [^{35}S]pro-OmpA into proteoliposomes was assayed by accessibility to added proteinase (2, 18). Each assay included purified SecA protein (50 μg/ml). Typically 20% or 45% of the pro-OmpA translocated into native KM9 or D10 membrane vesicles, respectively.

Other Methods. Protein (38) and lipid phosphorus (39) were determined as described. SDS/PAGE, silver staining, and immunoblotting were as described previously (1, 26). For assay of SecY, samples were incubated for 5 min at 37°C in SDS sample buffer prior to electrophoresis (24).

RESULTS

Glycerol Stabilizes the Solubilized SecY Protein. The SecY protein forms insoluble aggregates when solubilized from membranes with the nonionic detergent OG (26). Polytopic integral membrane proteins are usually not stable in micelles formed by detergents with a short acyl-chain length (25, 28, 40). Phospholipids and osmolytes are often included during solubilization to protect against denaturation. We therefore solubilized *E. coli* membrane vesicles with 1.25% (wt/vol) OG and a mixture of *E. coli* phospholipid and egg yolk phosphatidylcholine in the presence or absence of 20% (vol/vol) glycerol. After solubilization, the extract was centrifuged for 30 min at 145,000 × g_{max}. In the presence of glycerol, most of the SecY protein was retained in the supernatant (Fig. 1*A*, lane 7, supernatant; lane 9, pellet). Further centrifugation (120 min at 145,000 × g_{max}) revealed the presence of slowly sedimenting complexes of SecY (lane 8, supernatant; lane 10, pellet). Leader peptidase, another integral membrane protein, remained soluble under these conditions (Fig. 1*B*). As shown previously (26), almost all the SecY protein (Fig. 1*A*) and a substantial amount of leader peptidase (Fig. 1*B*) were found in the pellet fractions (lanes 4 and 5) in the absence of glycerol. Glycerol also affected the recovery of other membrane proteins as detected by silver-stained gels (data not shown) and protein assay (Table 1). Under all extraction conditions, phospholipid was almost completely recovered in the supernatant (Table 1). The insoluble fraction after OG extraction contained aggregated, partially delipidated membrane proteins.

SecY Protein Is Solubilized in a Nonaggregated Form. The SecY protein, extracted with OG in the presence of glycerol and phospholipids, was analyzed by gel filtration on an FPLC Superose 12 (Pharmacia) column. It migrated as a single peak with an apparent M_r of ≈58,000 (Fig. 2) and sedimented in a glycerol gradient with an apparent M_r of ≈66,000 (data not shown). Although these values are higher than the M_r of 49,000 predicted from the sequence of the *secY* gene (41, 42), these M_r estimates include any bound lipid, detergent, or other protein. We conclude that the solubilized SecY protein is in a true mixed micellar solution.

Reconstitution of Translocation Activity. Proteoliposomes were formed from the extracts by dialyzing to remove the detergent and then were assayed for their ability to substitute for membrane vesicles in the translocation ATPase reaction (16). The translocation ATPase requires SecA protein, membranes with a functional SecY protein, acidic phospholipids, and translocation-competent pro-OmpA or other precursor proteins (13, 16–18). To reduce the ATPase activity associated with the membranes, we used *E. coli* strain KM9, which lacks F_0F_1-ATPase (1). Reconstituted proteoliposomes were

Table 1. Reconstitution of the translocation ATPase

Test system	Centrifugation time,* min	Protein recovery, %	Phospholipid recovery, %	Translocation ATPase† Specific activity‡	Translocation ATPase† Yield, %
Inner membrane vesicles, KM9	—	100	—	148	100
Proteoliposomes,	—	100	100	72	48.6
no glycerol	30	44	98	33.5	10.0
during extraction	120	26	93	6	1.0
Proteoliposomes,	—	100	100	99.5	67.2
plus glycerol	30	95	92	96	61.6
during extraction	120	97	96	48	31.5

*At 145,000 × g_{max} in a Beckman Airfuge at 4°C.
†Difference between the rate of ATP hydrolysis with and without addition of pro-OmpA (40 μg/ml).
‡pmol/min per μg of protein.

supplemented with purified SecA protein, and the pro-OmpA-stimulated ATPase activity was measured. The presence of glycerol during detergent extraction dramatically improved the recovery of translocation ATPase activity that could be reconstituted from the soluble fraction (Table 1).

The translocation of pro-OmpA into the proteoliposomes was assayed by its inaccessibility to added proteinase K. Only proteoliposomes that had been reconstituted from the OG extract with glycerol retained a significant ability to translocate pro-OmpA in an energy-dependent manner. About 3.5% of the input pro-OmpA was translocated into proteoliposomes (Fig. 1*C*, lanes 5–8) and was partially processed to OmpA (the band below the pro-OmpA). Proteoliposomes reconstituted from an OG extract prepared without glycerol failed to translocate pro-OmpA (lanes 1–4).

Phospholipids Are Essential During Solubilization for Recovery of Translocation Activity. Membrane vesicles were solubilized with OG in the presence of glycerol and various amounts of phospholipid. After ultracentrifugation, phospholipid was added to the extract so that all reconstitutions were done with the same amounts of phospholipid. The phospholipid concentration during detergent extraction had little effect on the recovery of translocation ATPase activity (Fig. 3*A*, ●) or SecY protein (Fig. 3*B*). In contrast, the omission of phospholipid during extraction completely blocked recovery of energy-dependent pro-OmpA translocation into the proteoliposomes (Fig. 3*C*). This result suggests that translocation requires components or conditions beyond those needed for the translocation ATPase and that the reconstituted translocation activity is not due to residual nondisrupted membrane vesicles.

Authenticity of Pro-OmpA Translocation into Proteoliposomes. Efficient pro-OmpA translocation required ATP (Fig. 4*A*, lane 1 vs. lane 2). GTP (lane 5) and the nonhydrolyzable ATP analog adenosine 5′-[γ-thio]triphosphate (lane 6) could not replace ATP. Translocation of pro-OmpA was efficient at 40°C (lane 2) but did not occur at 0°C (lane 3). Unless stabilized by chaperone proteins (16), pro-OmpA rapidly oligomerizes and loses its translocation competence and ability to stimulate the translocation ATPase (ref. 13; S. Lecker and W.W., unpublished data). This translocation-incompetent form of pro-OmpA is neither a substrate of the reconstituted translocation ATPase (data not shown) nor translocated into proteoliposomes (Fig. 4*A*, lane 4). The SecA protein is essential for the translocation of pro-OmpA into proteoliposomes (Fig. 4*B*; lane 2, no SecA; lane 4, with SecA). When proteoliposomes were solubilized with 1.25% OG, the translocated pro-OmpA was accessible to protease (lane 5). The SecY protein is also required for translocation. A SecY-thermosensitive strain (CJ107) was exposed to the nonpermissive temperature and then used to prepare membrane vesicles. These membranes are unable to translocate pro-OmpA *in vitro* (32) or to support the translocation ATPase (16). Proteoliposomes prepared from these membranes were also defective for translocation (Fig. 4*C*, lane 4) and translocation ATPase (i.e., <9 pmol/min per μg of membrane protein). Proteoliposomes reconstituted from membranes derived from cells of this strain that were not exposed to the nonpermissive temperature showed normal levels of pro-OmpA translocation (Fig. 4*C*, lane 2) and translocation ATPase (i.e., ≈81.5 pmol/min per μg of membrane protein). Antibodies raised against the hydrophilic N-terminal domain of the SecY protein inhibit protein translocation *in vitro* (33) and block the translocation ATPase (16). Translocation ATPase (data not shown) and pro-OmpA translocation into the proteoliposomes were strongly inhibited by anti-SecY peptide antibody (Fig. 4*D*, lanes 1 and 3; control antibody in lane 5). Inhibition was to a large extent (though not completely) relieved by the N-terminal peptide

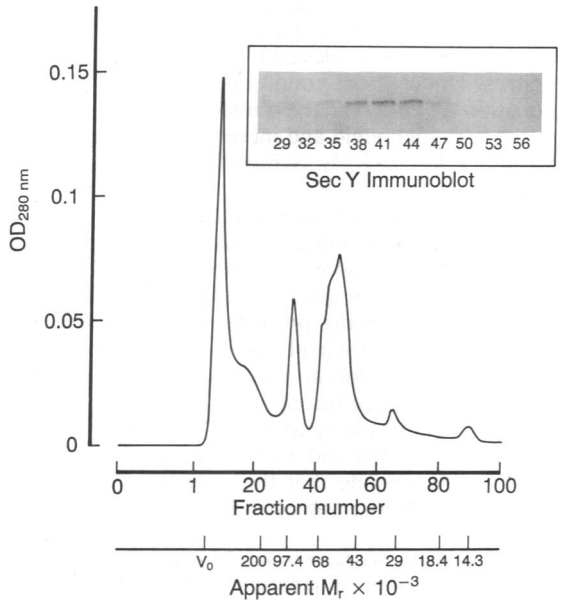

FIG. 2. Size-exclusion chromatography of a membrane extract prepared with OG, phospholipid, and glycerol. *E. coli* D10 membrane vesicle extract (200 μl, 155 μg of protein) was filtered through an FPLC Superose 12 (Pharmacia) column preequilibrated with 10% (vol/vol) glycerol and 1.25% (wt/vol) OG in buffer A at 0.5 ml/min. Elution was with the same buffer and 0.25-ml fractions were collected. Protein elution was monitored by absorbance at 280 nm. The column was standardized with 20 μg (200 μl) of each of the following proteins: myosin (M_r 200,000), phosphorylase *b* (M_r 97,400), bovine serum albumin (M_r 68,000), ovalbumin (M_r 44,000), carbonic anhydrase (M_r 29,000), β-lactoglobulin (M_r 18,400), and lysozyme (M_r 14,300). The exclusion volume of the column (V_o) was determined with Blue Dextran 2000. (*Inset*) Immunoblot of SecY peak fractions.

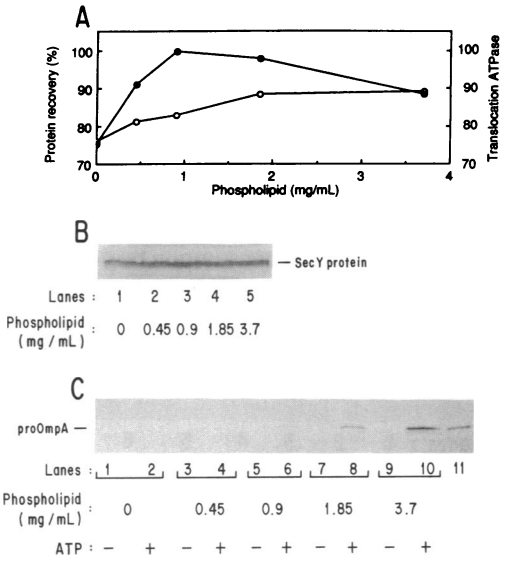

FIG. 3. Phospholipids are essential during solubilization for recovery of translocation activity. Membrane vesicles of *E. coli* KM9 were solubilized with OG and glycerol in the presence of various levels of phospholipid, as indicated. After centrifugation, supernatant fractions were assayed for total protein (*A*, ○) and SecY (*B*). Samples were supplemented with phospholipid to a final concentration of 3.7 mg/ml, reconstituted by detergent dialysis, and assayed for translocation ATPase (*A*, ●) and pro-OmpA translocation (*C*) in the absence or presence of ATP (4 mM) as indicated. Lane 11 represents a 2% [^{35}S]pro-OmpA standard. Densitometry showed that 0.6%, 1.4%, and 6.9% of the input pro-OmpA was translocated in lanes 6, 8, and 10, respectively. Translocation ATPase activity (*A*) is expressed in pmol/min per μg of membrane protein.

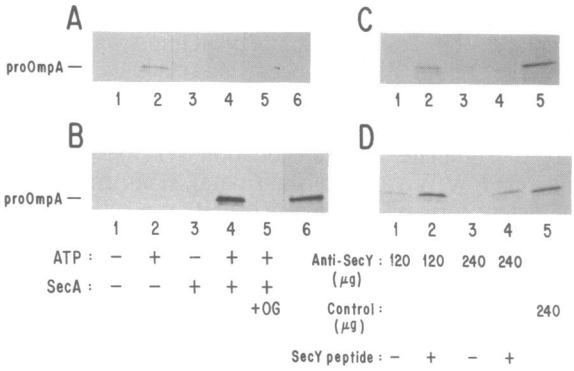

FIG. 4. Authenticity of pro-OmpA translocation into proteoliposomes. (*A*) Proteoliposomes derived from membranes of *E. coli* KM9 were assayed for pro-OmpA translocation at 40°C (lanes 1, 2, and 4–6) or 0°C (lane 3) in the absence (lane 1) or presence (4 mM) of ATP (lanes 2–4), GTP (lane 5), or adenosine 5′-[γ-thio]triphosphate (lane 6). To obtain the translocation-incompetent conformation (lane 4), pro-OmpA was diluted 35-fold into translocation buffer and incubated for 60 min at 40°C prior to the assay of translocation. (*B*) Translocation of pro-OmpA into proteoliposomes (derived from membranes of *E. coli* D10) in the absence or presence of purified SecA (50 μg/ml) and ATP (4 mM) as indicated. For lane 5, 1.25% (wt/vol) OG was added prior to proteinase K digestion. Lane 6 contained 10% of the input [^{35}S]pro-OmpA. (*C*) Proteoliposomes were reconstituted from membrane vesicles of the SecY thermosensitive strain CJ107 incubated at the permissive temperature (lanes 1 and 2) or at the nonpermissive temperature (lanes 3 and 4) prior to harvest and were assayed for pro-OmpA translocation in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of ATP. Lane 5 contained 5% of the input [^{35}S]pro-OmpA. (*D*) Proteoliposomes prepared from membranes of *E. coli* D10 (5 μg of membrane protein) were incubated for 1 hr on ice in translocation buffer with anti-SecY IgG or control IgG in the absence or presence of SecY peptide, as indicated. Translocation of pro-OmpA was assayed in the presence of ATP.

(Fig. 4*D*, lanes 2 and 4). These results suggest that a functional SecY protein is required for efficient translocation of pro-OmpA into the proteoliposomes.

pmf Stimulates the Translocation of Pro-OmpA into Proteoliposomes. The *in vitro* translocation of pro-OmpA is initiated by a reaction that requires only ATP and completed by a step that is driven by either ATP or a pmf (43, 44). To study the effect of a pmf on translocation in the proteoliposomes, the detergent extract was co-reconstituted with the light-driven proton pump bR. Reconstitution by detergent dialysis followed by freeze–thaw–sonication preferentially yields bR proteoliposomes with an inside-out orientation (35, 36). Upon illumination, these proteoliposomes generate an everted pmf—i.e., $\Delta\psi$, inside positive, and ΔpH, inside acid. In the presence of ATP and a low concentration of SecA protein, the translocation activity of proteoliposomes bearing bR was stimulated by illumination (Fig. 5, lane 1, plus light; lane 5, no light). In the presence of the ionophores nigericin and valinomycin, translocation activity was almost completely reduced to the level observed with ATP alone in the dark (lanes 4 and 5). Collapse of the ΔpH by the addition of nigericin hardly affected translocation (lane 2). The activity was significantly reduced when the $\Delta\psi$ was dissipated with the ionophore valinomycin (lane 3). bR liposomes, reconstituted without the detergent-extracted membrane proteins, were unable to translocate pro-OmpA (lanes 7 and 8). Thus the stimulatory effect of the pmf seen with native membrane vesicles (45) can be restored in proteoliposomes.

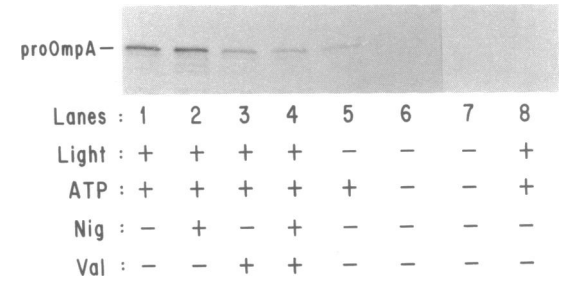

FIG. 5. Translocation of pro-OmpA into proteoliposomes is stimulated by pmf. *E. coli* KM9 membrane vesicles were solubilized with OG in the presence of glycerol and bR proteoliposomes. Pro-OmpA translocation activity of the co-reconstituted bR proteoliposomes and bR liposomes alone was assayed in the presence of purified SecA (5 μg/ml) and, where indicated, 2 mM ATP, 10 nM nigericin (Nig), and 100 nM valinomycin (Val). Tubes were sealed with a cover glass to prevent evaporation, and illuminated with a 150-W tungsten bulb. Ionophores were added as ethanolic stock solutions so that each reaction mixture included 2% (vol/vol) ethanol.

DISCUSSION

We have reported a means of solubilization of *E. coli* membrane vesicles that allows the subsequent reconstitution of protein-translocation activity into proteoliposomes. The authentic character of the reconstituted translocation reaction was shown by its dependence on ATP, SecA protein, a functional SecY protein in the membrane, a translocation-competent conformation of the precursor, and the pmf.

Glycerol (27, 28) and phospholipids (29, 40), present during solubilization of the membranes with OG, prevent the irreversible aggregation of SecY and minimize the loss of function upon reconstitution. The solubilized proteins are in true mixed micellar solution since (*i*) the ability to reconstitute pro-OmpA translocation activity from the extract is retained after centrifugation at 145,000 × g_{max}, (*ii*) the SecY protein partitions as a symmetric, included peak on a gel filtration column, and (*iii*) substantial purification of the SecY protein in this extract can be achieved by various chromatographic procedures. Under these conditions, the SecY protein, the translocation ATPase, and the translocation activity itself are recovered with high yield and increased specific activity (L. Brundage, J. Hendrick, E. Schiebel, A.J.M.D., and W.W., unpublished data).

The solubilization and reconstitution procedure provides an avenue to isolate the integral membrane proteins involved in the protein-translocation reaction. The pure components may allow several major questions of protein translocation to be addressed: How many proteins are involved in translocation, and what are their physical and functional relationships? Do precursor proteins traverse the membrane through a proteinaceous pore or through the lipid bilayer, and what is the role of the acidic phospholipids (18, 46)? What is the role of energy, both as pmf and as ATP, in translocation? Other studies (44, 45) indicate that the requirement for ATP and SecA may precede the potential-dependent step.

Note Added in Proof. We have isolated from this extract a complex containing Sec Y and Sec E polypeptides that can be reconstituted into proteoliposomes to support authentic pro-OmpA translocation (L. Brundage, J. Hendrick, E. Schiebel, A.J.M.D., and W.W., unpublished work).

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